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14. ABSTRACT
The goal of this proposal is to develop a novel delivery system for siRNA therapeutics to enable a more targeted delivery approach than is currently possible. The proposed delivery system relies on two classes of gold nanoparticles each targeted to a separate surface marker and each class containing part of a construct that ultimately will assemble into the therapeutic complex after internalization and release. Only when both classes of gold nanoparticles bind the cell can the therapy be activated. This one year proof-of-principle project will demonstrate the proposed concept using a probe for imaging and knockdown of telomerase in breast cancer cells activatable only in cells which are both HER 2+ and EGFR+. As siRNA technologies evolve, such an approach might prove clinically valuable for sub- populations of women whose tumors are both HER2+ and EGFR+.

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TABLE OF CONTENTS

INTRODUCTION

In this project, we proposed a fundamentally new approach to targeted breast cancer therapeutics with the potential to dramatically improve specificity, reducing unwanted side effects. Here, we review our original proposal. In the body of the report, we describe what we learned while carrying out this work. Currently, therapeutic regimens for treatment involving more than one molecular target (HER2, EGFR, VEGF, etc.) sequentially treat the cancer based on one *OR* another of the chosen targets. Rapidly increasing understanding of the molecular alterations of breast cancer coupled to constantly improving technologies for characterizing individual patient tumor biology suggest it is an appropriate time to consider how to improve the specificity of breast cancer therapy. The goal of this work was to propose a new type of therapy activated only in tumors presenting both a first *AND* second molecular target. Chemotherapy today relies on a combined effort (an *OR* approach) to eradicate cancer cells with each drug having its own target and toxicity. We proposed a novel delivery method (an *AND* approach) in which two types of AuNPs must be present and interacting for successful delivery to occur. This would drastically lower toxicity to non-specific sites since the therapy could only be activated in the presence of both targets. We planned to use two classes of small AuNPs for delivery of the therapy: one class carries the sense strand and the second the anti-sense strand. Each class is targeted to a distinct molecular marker. The siRNA therapy can only be activated in cells expressing both markers since only one of the needed strands is carried by the AuNP which binds to an individual target. After binding and endosomal escape, the sense and anti-sense strands should anneal and the complete siRNA may then be released to allow silencing. We planned to demonstrate siRNA mediated knockdown of telomerase in SKBR3 breast carcinoma cells which are both HER2+ and EGFR+. The use of two classes of AuNPs provides not only an efficient carrier and activation mechanism but also a method to track delivery and monitor transfection efficiency of each component. The general approach we describe, if successful, would offer a broadly applicable method to improve the specificity of gene therapy. It is critical such methods are developed if gene therapy techniques are to become clinically viable in the future. The specific approach would be applicable for any form of siRNA therapy in women whose tumors are HER2+ and EGFR+. However, the greater significance of the approach is that it could be used in a patient-specific manner with a combination of molecular targets most suited to the individual patient's tumor biology.

PROGRESS REPORT BODY

We have begun preparing manuscripts on some of the work described below. Some text and figures below are from these manuscript drafts that have not yet been submitted for publication.

Our proposed dual GNP siRNA delivery system consists of two gold nanoparticles conjugated with two different antibodies tow ard specific surface proteins on the cancer cell. Of two nanoparticles, one is conjugated with the sense strand of the siRNA, and the other one with th e anti-sense strand. B oth particles m ust be present in the cytosol to form a complete siRNA. Both particles ar e conjugated with cell penetrating peptides (CPPs) to induce release from the endosom es. When the NIR is shined on the gold particles, heat is generated that triggers the releas e of the sense or antisen se strand of the siRNA fr om the gold particles. After the strands are released , they will hybridize in the cytosol to form a functional siRNA. To promote hybridization of the sense and antisense strands, we propose using a hairpin/molecular beacon shaped design (Figu re 1). With our light trigger system, we should be able to con trol th e position and the tim e of siRNA rele ase, lim iting any unwanted sid e effects from this therapy

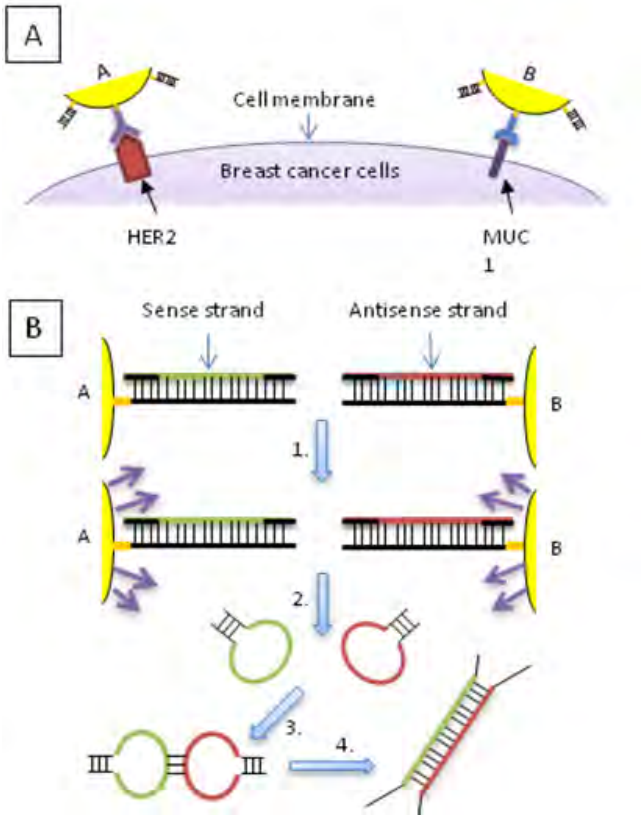

Figure 1.

Specific Aim 1:

The first portion of the synthesis process was preparation of the gold nanoparticles (AuNPs). A major issue to consider in this step is m inimizing toxicity. We have found the standard citrate based approaches to produce undesi rable levels of cytotoxicity ev en though this is the standard protocol used for nanom edicine work. We kn ew this would be a problem for the project proposed here. Thus, over the past year we creat ed an alternative approach to production of high quality AuNPs which we desc ribe here. We believe this approac h will be highly valuable for other work requiring AuNPs for biological a pplications as well. AuNPs were synthesize d by CO reduction, a technique not used previous ly for this purpose. AuNPs with average diameter nanoparticles ranging from 4 to 52nm were prepared. (These sizes are m ost relevant to this project because we need internalization of the AuNPs for delivery of the siRNA and it is easiest to d eliver AuNPs in th is size regim e). A se t of solutions consis ting of varying

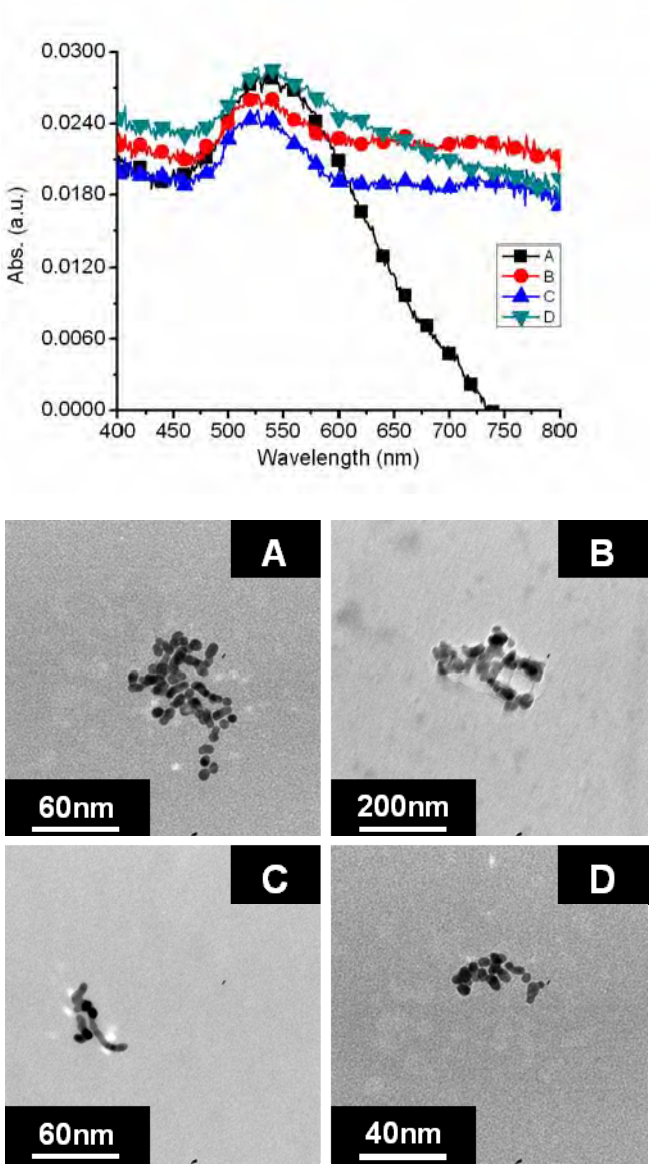

Figure 2. AuNP synthesis. UV-visible extinction spectra of nanoparticles synthesized from a chloroauric acid concentration of 0.01mM aerated at flow rates of 16.9, 25.5, 37.0, and 42.9 mL/min corresponding to A, B, C, and D, respectively with accompanying TEM micrographs.

concentrations of $HAuCl_4$ ranging from 0.01mM up to 0.09mM in 0.01mM increments were used. Each $HAuCl_4$ concentration was duplicated to ensure reproducibility. For each $HAuCl_4$ concentration five 40mL samples were prepared. Each sample was aerated at different flow rates controlled by a control valve. The five solutions were exposed to CO gas at flow rates of 16.9, 25.45, 31.59, 37.0 and 42.9 mL/min respectively. The effect of stirring speed was examined and it was found that the revolution per minute (rpm), by which the solution was stirred, plays a role in particle size and morphology. The optimum stir speed was found to be 500 rpm. For the following discussion each solution, during synthesis, was constantly stirred at a rate of 500 rpm unless noted otherwise. The solution temperature, prior to aeration, was maintained between 20 and 22°C.

To illustrate the effects of CO gas flow injection rates on nanoparticle synthesis a solution consisting of a low chloroauric concentration was used. Nanoparticles were synthesized from an aqueous solution of $HAuCl_4$ acid at a concentration of 0.01mM. Even at this lower concentration the extinction spectra is clearly visible and well formed as evident in Figure 2. A smoother more pronounced spectrum was generated at the minimum flow rate of 16.9 mL/min when compared to the other injection flow rates. As the flow rate was increased from 16.9 to 42.9 mL/min the change in spectral symmetry was clearly visible. TEM micrographs of the corresponding nanoparticles are displayed in Figure 2. The gas injection flow rate of 16.9 mL/min produced individual nanoparticles compared to the other injection rates. The nanoparticles produced by the 16.9 mL/min flow rate ranged in size from 5 to 11 nm in diameter. A flow rate of 25.45 mL/min,

Figure 2B, produced nanoparticle aggregates and irregularly shaped particulate matter. Nanoparticles synthesized at a flow rate of 31.59 mL/min consisted of aggregated particle chains. A CO flow rate of 37 mL/min (Figure 2C) resulted in aggregated particle chains similar to that of nanoparticles produced at a flow rate of 25.45 mL/min. The particle aggregation in Figure 2B and 2D was evident by the broad spectral band. As the flow rate increased to 42.9 mL/min the nanoparticles became elliptical in shape and very polydispersed. The nanoparticle sizes, when aerated at 42.9 mL/min, ranged from 5 to 40nm in diameter with some aggregated particles and this size distribution is reflected in the broad spectral band. Increasing the chloroauric acid concentration reduced the polydispersity of the nanoparticles yet the gas injection flow rate continued to influence the AuNP size distribution profiles.

By employing a combination of gold polymer reduction and gold hydrolyzed polymer reduction particles sizes from ~4nm to 100nm can be synthesized. Figure 3 shows a TEM micrograph illustrating the different sizes available using CO as a reducing agent. 3A, 3B, 3C, and 3D are TEM images of AuNPs synthesized without the addition of K_2CO_3. 3E and 3F are AuNPs synthesized from a hydrolyzed solution of aqueous $HAuCl_4$ via the addition of K_2CO_3. The corresponding sizes of the AuNPs are 4, 6, 15, 25, 50, and 100nm with standard deviations of 7, 13, 8, 8, 10, and 11%, respectively. These particles span the size regime over which internalization of AuNPs is achieved as required for siRNA delivery. Our first experiments used particles of similar sizes to those shown in Figure 3D. As noted later, we found it difficult to create stable functionalized AuNPs at this size and are presently conducting experiments with smaller particles.

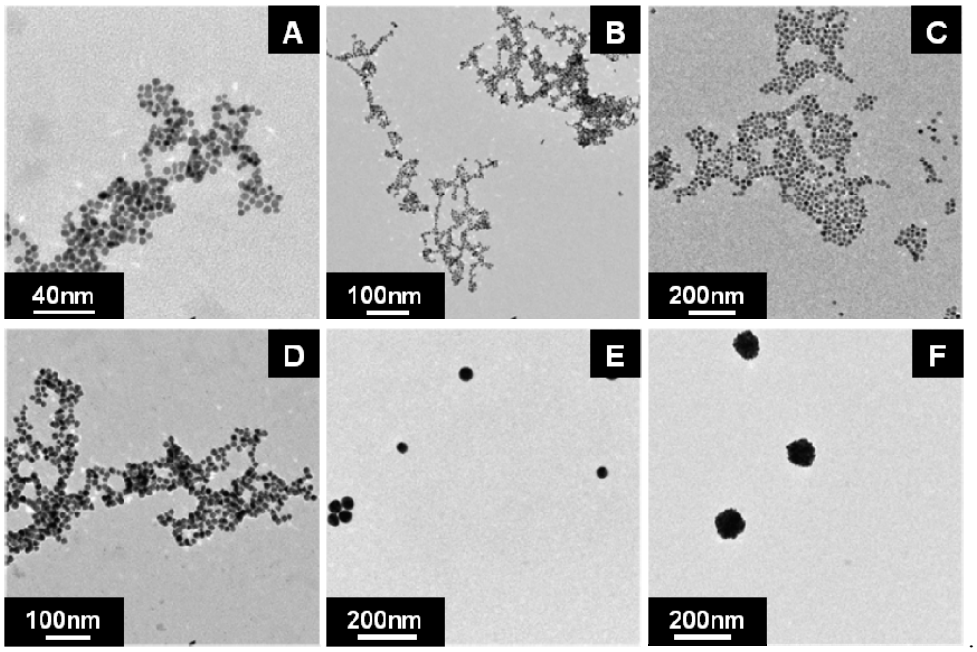

Figure 3. TEM images of AuNPs synthesized by CO reduction of $HAuCl_4$. A, B, C, and D are TEM images of AuNPs synthesized without the addition of K_2CO_3. E and F are AuNPs synthesized from a hydrolyzed solution of aqueous $HAuCl_4$ via the addition of K_2CO_3. The corresponding sizes of the AuNPs are 4, 6, 15, 25, 50, and 100nm.

After preparation and charac terization of the AuNPs to be used for original experiments which will be described in a paper in preparation, m uch of our work this year focused on refining the orig inal strategy proposed to synthesize the structures described. In our original proposal we proposed structures as indicated in Figure 4. However, we did not describe a d etailed approach to synthesis of these particles. In atte mpting to fabric ate these particles, we discovered that it was prohibitive ly challenging to create a particle with all of the necessary com ponents (siRNA strands, an tibodies for targeting, and peptides for internalization while keeping a go ld nanoparticle stable. Thus, we have developed a new approach to synthesis which we describe in Figure 5. Comparing Figure 3 and Figure 4, note that the same general components are used to deco rate the pa rticle su rface (siRNA, antibodies, and peptide) but the structure is a bit different in that all of the antibody is on one side and all of the endosom al escape peptides on another. Also, the siRNA are shown in linear r ather than loop form (see res ults figure in Specific Aim 2 for in itial knockdown results comparing loop and straight configurations). Although we have not been able to com pletely generate th ese pa rticles yet, we believ e th is new stra tegy is f easible and will allow us to ultimately create these particle s. We describe the plan we have com e up with below. We initially planned to use ~30 nm AuNPs in this work. However, we had dif ficulty in attempting to stab ilize particle s of this size . We have found it m ost fe asible to stabilize AuNPs with oligonucleotides attached for either sm all (< 15nm) or larger (~100 nm) particles.

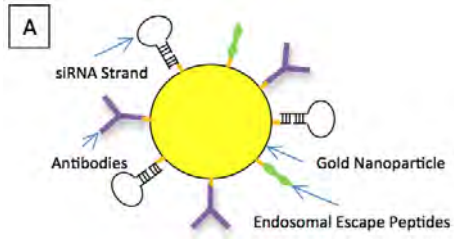

Figure 4.

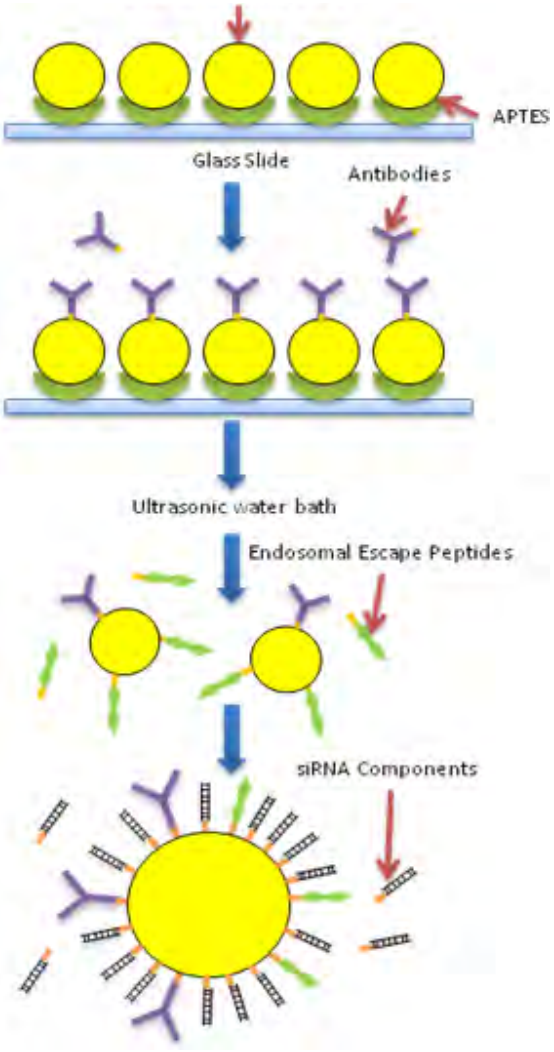

Figure 5.

In the new approach we propose, AuNP will be placed on3-Am inopropyltriethoxysilane (APTES) coated slides to form a monolayer.

Antibodies attached with PEG-linkers will then be added to the AuNP-slide. The APTES will only allow antibodies to bind on half of the AuNP surface. The AuNP-Ab complex will be released from the APTES using an ultrasonic water bath. TAT proteins with SPDP are then added to the AuNP-Ab solution after reduction by dithiothreitol (DTT). This forms the ***primary complex*** (AuNP-Ab-TAT) with antibodies: anti-HER2 or anti-EGFR. The *primary complex* may not be stable or protected from pH change or high salt solutions since the antibodies and TAT protein binding do not form a tight layer. There will be many gaps in between the proteins, exposing bare gold surfaces. siRNA sense and antisense oligos will be added to fill in the gaps. Because the sense and antisense strand need to anneal under physiologic temperatures, we will compare our loop designs as shown below with single strands to find the most efficient method (Figure 6). The hairpin/loop structure should allow a much higher hybridization of the sense and antisense strands and may increase the half-life of the oligo within the cytosol, after being released from the AuNP surface. For preliminary results, we are using telomerase siRNAs with antisense strand sequence: 5'-CUCAGUUAGGGUUAGACAAUU-3'[21]. Compliment strands will have 5' C6 thiol modification. The thiol-modified oligos will be uncapped using a solution mix of dithiothreitol (DTT) in sodium phosphate buffer. The mixture will run through a column to remove DTT and byproducts. Final uncapped oligos will be eluted and concentration determined by measuring OD260. The uncapped oligos will be added to the primary complex following protocol by Taton (Taton, 2002). Compliment-AuNP-Ab-TAT complexes can be extracted through centrifugation. Sense or antisense strands will be then added to the complex solution at 50 °C for 30 minutes to allow annealing of the partial siRNA to the compliment strands on the GGS. The ***final complexes*** (partial siRNA-AuNP-Ab-TAT) can be harvested through centrifugation. Two important factors will need to be characterized. First, we will also do a salt assay to evaluate the stability of the GGS. The *final complexes* will be incubated in PBS and 0.1 M NaCl/10 mM sodium phosphate buffer for 2 hours to test for integrity. A decrease in plasmon peak of greater than 15% will be defined as not stable complexes. Secondly, in order to see the amount of RNA that can be released by the final complex, we will do a ramp test of increasing light power to find the minimum energy needed to obtain maximum RNA release. *Final complexes* will be added to a glass slide coated with HER2. Various power and exposure times will be used on the complexes. The increase in OD260 will determine the amount of DNA released from the GGS. Using these results, we will be able to determine the best ratio of antibody:TAT:Oligo for stable and effective siRNA delivery.

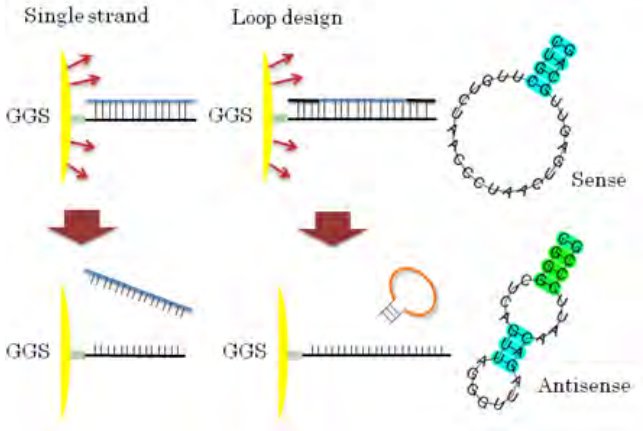

Figure 6.

Specific Aim 2:

Because we found the synthesis in Aim 1 more challenging than originally expected, we began a modified version of Aim 2 so we could start this part of the project while Aim 1 was underway. Our main goal in Aim 2 was to assess the feasibility of the overall idea we proposed. Figure 7 in a single graph shows both the potential and limitations of the strategy we proposed. This graph plots telomerase expression relative to expression in the control cells for the two geometries we have considered (loop "L", Figure 4, our original configuration, and straight "S", Figure 5, our revised configuration). These experiments were conducted using SKBR3 breast carcinoma cells and evaluation knockdown of telomerase using a trapeze assay. The idea of the project was that one would increase specificity of knockdown by separating the sense and antisense strands using two nanoparticles to carry them, each carrying either sense or anti-sense while targeted to a separate marker. The most significant challenge with the idea as proposed is that we found some degree of knockdown occurs in all cases (including when a strand or loop is delivered individually without the other half of the siRNA). The idea as proposed assumed minimal to low level of knockdown when using individual strands or individual loops. Our results so far suggest that it is possible to achieve knockdown using a strategy in which the sense and antisense are delivery separately (as Figure 7 demonstrates) but that it is not realistic to suggest knockdown would only occur in cells positive for both markers. What would be likely is that the degree of knockdown would be higher in cells with both markers rather than other cells presenting only one marker or neither marker. Thoroughly evaluating this would require a more stable experimental system than that used here as well as more fully optimized siRNA.

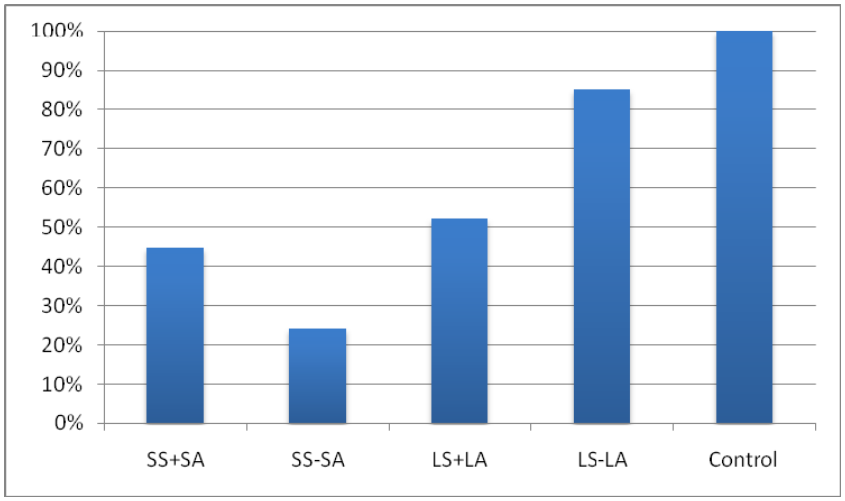

Figure 7. Comparison of telomerase knockdown in SKBR3 breast carcinoma cells for straight (S) and loop (L) designs. All combinations considered resulted in some level of telomerase knockdown. SS = single stand sense, SA = single send antisense, LS = loop sense, LA = loop antisense. The "+" indicates components added together using lipofectamine for delivery *post* incubation and the "-" indicates components added together using lipofectamine for delivery *prior* to incubation.

KEY RESEARCH ACCOMPLISHMENTS

- New method for synthesis of gold nanoparticles does not require the use of citrate used in most current methods for synthesizing gold nanoparticles in this size regime. We believe this will result in reduce cytotoxicity relative to current approaches and will carefully assess this in the future. A manuscript on this work has been completed and will be submitted during Fall 2010.

- New synthesis strategy was developed to pattern antibodies onto a fraction of the nanoparticle surface leaving a fraction free for TAT with a final step to add siRNA.

- Preliminary biological results suggest telomerase knockdown is achieved delivering the strands separately as proposed. However, the level of knockdown present from the antisense strand or loop alone was more significant than anticipated and requires some modification of the original concept.

REPORTABLE OUTCOMES

Presentations:

- We plan to submit an abstract on this work to the 2011 Era of Hope Breast Cancer Meeting. Abstract submission for this meeting is not yet open. The planned submission will be:

  Lin, A., Young, J., and Drezek R. Changing the OR to AND in Dual Targeted Therapeutics for Breast Cancer. CDMRP Era of Hope Breast Cancer Meeting. Summer 2011.

Proposals:

- We have jointly applied to a NSF program (IIP, Division of Industrial Innovations and Partnerships) to fund future work on this idea (submitted Spring 2010). Specifically, we have proposed to work together with a company with expertise in labeling oligonucleotides to assist in subcellular visualization of siRNA delivery using the various schemes we have developed. Although I have not yet received funding (we are the subaward recipient and the SBIR company is the prime), the company has indicated that this work will be funded. This will allow us to continue to develop this concept further.

CONCLUSIONS

Future applications if the system described can be realized (even if knockdown in regions without both markers is not fully prevented) are substantial. This system could help treat individuals with treatment resistant tumors, could potentially lower metastasis risks, and even personalize cancer treatment depending on the expressed surface proteins and the siRNA used. In the shorter term, we envision the system to improve adjuvant or systemic chemotherapy for superficial breast cancers and diminish recurrence rates post tumor excision by treating tumor margins. In summary, with signficant further development, it is hoped this system could help bring silencing gene therapy from bench top to bedside.

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Taton TA. Preparation of gold nanoparticle-DNA conjugates. *Current protocols in nucleic acid chemistry*. 2002; Chapter 12:Unit 12.2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18428889.